Activation of MYCN by trastuzumab.

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Trastuzumab functions by interactions between the F_{ab} domain of trastuzumab and the extracellular domain of the human epidermal growth factor receptor 2 (HER2)¹. The signals that are transduced following these interactions, and the transcriptional consequences of these interactions are less well understood; we studied these responses by mining published microarray and multiplexed gene expression profiling datasets²⁻⁵. We observed significantly increased expression of MYCN in the primary tumors of patients with breast cancer treated with trastuzumab. MYCN was also differentially expressed upon a single, brief exposure to trastuzumab, when comparing the same tumors before and after treatment, indicating MYCN is activated by trastuzumab. Transcription of another MYC family protein, MYC, was decreased as opposed to increased when comparing the tumors of patients who did not develop brain metastases based on trastuzumab treatment history. However, in patients who did develop brain metastases following trastuzumab treatment, we observed significantly increased levels of MYC. MYC expression level might predict which patients will develop brain metastases, or the MYC transcriptional response to trastuzumab might foretell brain metastatic response. In addition, we found that an inhibitor of the MYC gene expression program, MXD1^{6,7}, was present at significantly decreased levels in the tumors of patients treated with trastuzumab. MYC family proteins function as oncoproteins and the evidence provided here, demonstrating increased expression of Myc family oncoproteins and decreased expression of a Myc inhibitor, should elicit broader assessment of the transcriptional responses to trastuzumab in patients with breast cancer.

Keywords: breast cancer, trastuzumab, MYC, MYCN, MXD1, the MYC family of oncoproteins, central nervous system metastasis, brain metastasis.

Trastuzumab is utilized in adjuvant breast cancer and for the treatment of metastatic breast cancer. Trastuzumab is a targeted monoclonal antibody against the human epidermal growth factor receptor 2, HER2, encoded by the ERBB2 gene. While the mechanism of action ascribed to trastuzumab is thought to involve binding of the F_{ab} domain of trastuzumab to the extracellular portion of HER2¹, the transcriptional response to signals transduced following such binding is less well understood. We mined published microarray and multiplexed gene expression datasets²⁻⁵ to understand the transcriptional response to trastuzumab in the tumors of patients with breast cancer, segregating and comparing patients based on those who did or did not develop brain metastases following treatment with trastuzumab. We discovered concerning evidence demonstrating increased expression of members of the MYC family of oncoprotein, in conjunction with decreased expression of a MYC family inhibitor^{6,7}, in the tumors of patients treated with trastuzumab.

Methods

We used datasets GSE38057², GSE22226³, GSE75685⁴ and GSE114082⁵, for this global differential gene expression analysis in conjunction with GEO2R. GSE38057 was generated using DASL Human Cancer Panel by Probe technology with either $n=11$ primary breast tumors from patients treated with induction chemotherapy and/or hormone therapy without trastuzumab who had not developed brain metastases at time of tissue sampling and $n=35$ primary breast tumors from patients treated with induction chemotherapy and/or hormone therapy in addition to trastuzumab who had not developed brain metastases at time of tissue sampling, or $n=6$ primary breast tumors from patients with breast cancer not treated with trastuzumab who had developed brain metastases and $n=35$ primary breast tumors from patients with breast cancer who had developed brain metastases at time of tissue sampling; analysis was performed using platform GPL5826. GSE22226 was generated using Whole Human Genome Oligo Microarray G4112A technology with $n=118$ primary breast tumors from patients treated with chemotherapy and $n=11$ primary tumors from patients treated with chemotherapy and trastuzumab, analysis performed using platform GPL1708. GSE75685 was generated using Whole Human Genome Microarray 4x44K technology with $n=41$ primary breast tumors from patients treated with chemotherapy and $n=11$ primary tumors from patients treated with chemotherapy and trastuzumab, analysis performed using platform GPL4133. GSE114082 was generated using Illumina HumanHT-12 WG-DASL V4.0 R2 expression beadchip technology with $n=17$ primary breast tumors from patients before trastuzumab treatment and $n=17$ primary tumors from patients after trastuzumab treatment, analysis performed using platform GPL14951. The Benjamini and Hochberg method of p -value adjustment was used for ranking of differential expression but raw p -values were used to assess statistical significance of global or multiplexed differential expression. Log-transformation of data was auto-detected, and the NCBI generated category of platform annotation was used. A statistical test was performed to evaluate whether MYC, MYCN, or MXD1 gene expression was significantly between primary tumors from patients treated or not treated with trastuzumab using a two-tailed, unpaired t-test with Welch's correction. We used PRISM for all statistical analyses of differential gene expression in human breast cancer (Version 8.4.0)(455).

References

We mined published microarray and multiplexed gene expression profiling datasets²⁻⁵ to discover genes associated with trastuzumab treatment in the primary tumors of patients with breast cancer.

MYCN is expressed at significantly higher levels in the primary tumors of patients with breast cancer who did not develop brain metastases when comparing tumors based on trastuzumab treatment.

MYCN, also known as N-Myc, was among the genes whose expression was most significantly different when comparing the tumors of patients with breast cancer who did not develop brain metastases at time of tissue sampling based on treatment with trastuzumab². When sorting each of the genes based on significance of difference in expression between the primary tumors of patients who had not developed brain metastases at time of tissue sampling based on treatment with trastuzumab, MYCN ranked 120 out of 1506 total transcripts (Table 1), equating to 92% differential expression. Differential expression of MYCN in the primary tumors of patients with breast cancer who had not developed brain metastases at time of tissue sampling based on treatment with trastuzumab trended towards statistical significance (Table 1: $p=0.093808$). MYCN was expressed at significantly higher levels in the tumors of patients treated with trastuzumab as compared to the tumors of patients not treated with trastuzumab (Figure 1; $p=0.0196$). We also asked whether we could observe changes in MYCN expression in patients who did develop brain metastasis, when comparing patients treated with trastuzumab as with those not treated with trastuzumab². While the dataset probed was limited to 1506 transcripts, we were still able to detect a positive fold change of MYCN expression in patients treated with trastuzumab that had developed brain metastasis as compared to patients not treated with trastuzumab that did develop brain metastasis (data not shown). Thus, in patients with breast cancer, both in patients that did and did not develop brain metastasis, trastuzumab treatment was associated with real quantitative increases in primary tumor MYCN transcript levels as compared to patients not treated with trastuzumab.

We mined a second microarray dataset to determine if we could validate differential expression of MYCN in breast cancer patients treated with trastuzumab and to ascertain whether lack of statistical significance of MYCN differential expression was due to low transcript number resulting from technology utilized in the first dataset analyzed. MYCN was again among the genes most differentially expressed transcriptome-wide when comparing the primary tumors of patients treated with trastuzumab and that of patients not treated with trastuzumab (Table 2). When sorting each of the genes expressed in primary tumors of the breast based on significance of changes in expression when comparing tumors of the breast from patients treated with trastuzumab or not, MYCN ranked 934 out of 44290 total transcripts (Table 2). Differential expression of MYCN in the primary tumors of breast cancer patients treated with trastuzumab was statistically significant (Table 2: $p=7.92E-03$).

MYC is expressed at lower levels in the primary tumors of patients with breast cancer who did not develop brain metastases when comparing tumors based on trastuzumab treatment.

MYC was among the genes whose expression was most significantly different when comparing the tumors of patients with breast cancer who did not develop brain metastases at time of tissue sampling based on treatment with trastuzumab. When sorting each of the genes based on significance of difference in expression between the primary tumors of patients who had not developed brain metastases at time of tissue sampling based on treatment with trastuzumab, MYC ranked 143 out of 1506 total transcripts (Table 3). Differential expression of MYC in the primary tumors of patients with breast cancer who had not developed brain metastases at time of tissue sampling based on treatment with trastuzumab was not deemed statistically significant (Table 3: $p=0.114767$), despite equating to 90.5% differential expression. MYC was expressed at lower levels in the tumors of patients treated with trastuzumab as compared to the tumors of patients not treated with trastuzumab, but this difference was not statistically significant (Figure 2; $p=0.1518$).

We mined a separate microarray dataset to validate differential expression of MYC in patients treated with trastuzumab. MYC was again among the genes most differentially expressed transcriptome-wide when comparing the primary tumors of patients treated with trastuzumab and that of patients not treated with trastuzumab (Table 4). When sorting each of the genes expressed in primary tumors of the breast based on significance of changes in expression when comparing tumors of the breast from patients treated with trastuzumab or not, MYC ranked 1159 out of 45220 total transcripts (Table 4). Differential

expression of MYC in the tumors of breast cancer patients treated with trastuzumab was statistically significant (Table 4: $p=1.16E-03$).

MXD1, an inhibitor of the Myc gene expression program, is expressed at significantly lower levels in the primary tumors of patients with breast cancer who did not develop brain metastases when comparing tumors based on trastuzumab treatment.

MXD1 was among the genes whose expression was most significantly different when comparing the tumors of patients with breast cancer who did not develop brain metastases at time of tissue sampling based on treatment with trastuzumab. When sorting each of the genes based on significance of difference in expression between the primary tumors of patients who had not developed brain metastases at time of tissue sampling based on treatment with trastuzumab, MXD1 ranked 1 out of 1506 total transcripts (Table 5), equating to 99.9% differential expression. Differential expression of MXD1 in the primary tumors of patients with breast cancer who had not developed brain metastases at time of tissue sampling based on treatment with trastuzumab was statistically significant (Table 5: $p=0.000129$). MXD1 was expressed at significantly lower levels in the tumors of patients treated with trastuzumab as compared to that of patients not treated with trastuzumab (Figure 3; $p=0.0076$).

We mined a separate microarray dataset to validate differential expression of MYC in patients treated with trastuzumab. MXD1 was again among the genes most differentially expressed transcriptome-wide when comparing the primary tumors of patients treated with trastuzumab and that of patients not treated with trastuzumab (Table 6). When sorting each of the genes expressed in primary tumors of the breast based on significance of changes in expression when comparing tumors of the breast from patients treated with trastuzumab or not, MXD1 ranked 216 out of 44290 total transcripts (Table 6). Differential expression of MXD1 in the tumors of breast cancer patients treated with trastuzumab was statistically significant (Table 6: $p=1.08E-03$).

MYCN is among the genes whose expression changes most significantly upon a brief, single exposure to trastuzumab when comparing tumors from the same patient before and after trastuzumab administration.

Differential and increased expression when comparing patients treated with trastuzumab or not, in and of itself, does not purely indicate activation. We used a fourth dataset to compare tumors from the same patient who were treated with a single, brief exposure to trastuzumab to determine if MYCN was among the genes whose expression changed most significantly before and after trastuzumab treatment. In fact, this was the case: when sorting each of the genes expressed in the tumors of breast cancer patients based on change of MYCN expression before and after a single treatment with trastuzumab, MYCN was ranked 9472 out of 29377 total transcripts, equating to 67.8% differential expression. Differential expression of MYCN following a single administration of trastuzumab was statistically significant (Table 7: $p=4.5E-02$). Thus, together with data demonstrating significantly increased expression of MYCN, we conclude that MYCN is activated in the tumors of patients with breast cancer by treatment with trastuzumab.

MYC is expressed at higher levels in the primary tumors of patients with breast cancer when comparing tumors between patients who did develop brain metastases based on trastuzumab treatment.

Since both MXD1 and MYCN were expressed at significantly higher levels in the tumors of patients treated with trastuzumab but MYC was not, we asked whether patterns of differential expression of MYC family oncoprotein was different when comparing the tumors of patients who did develop brain metastases following treatment with trastuzumab. We found that in patients who did develop brain

metastases, MYC was again among the genes whose expression changed most significantly when comparing the tumors based on trastuzumab treatment. When sorting each of the genes based on significance of difference in expression between the primary tumors of patients who had developed brain metastases at time of tissue sampling based on treatment with trastuzumab, MYC ranked 9 out of 1506 total transcripts (Table 8), equating to 99.4% differential expression. Differential expression of MYC in the primary tumors of patients with breast cancer who had not developed brain metastases at time of tissue sampling based on treatment with trastuzumab was statistically significant (Table 8: $p=0.01484$). MYC was expressed at significantly higher levels in the tumors of patients treated with trastuzumab who had developed brain metastases (Figure 4: $p=0.0267$).

Discussion

We provided evidence here that breast cancer patients treated with trastuzumab display evidence of transcriptional activation of MYCN in primary tumors, that an inhibitor of MYC family proteins, MXD1, is expressed at significantly lower levels in the tumors of patients treated with trastuzumab, and that while MYC was expressed at lower levels in the tumors of trastuzumab-treated patients who had not developed brain metastases, MYC was expressed at significantly higher levels in the tumors of trastuzumab-treated patients who had developed brain metastases. These data in total point to aberrant activation of a Myc gene expression program in the primary tumors of breast cancer patients treated with trastuzumab, and suggest that Myc expression levels may predict which patients will develop brain metastases upon treatment with trastuzumab, as an indication of cooperative behavior with other, as of yet undescribed gene products, or that the MYC transcriptional response to trastuzumab might foretell brain metastatic response.

Targeted ectopic expression of MYCN results in the development of neuroblastoma in mice⁸. These data should be evaluated with an index of suspicion and serve as a warning signal for the potential for increased development of brain metastases in patients with breast cancer treated with trastuzumab⁹.

References

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Rank	ID	p-value	t	B	FC	Gene
120	GI_34147496-S-3	0.093808	1.70912	-4.23	2.15 ± 2.14	MYCN

Table 1: Differential expression of MYCN in primary tumors of breast cancer patients treated with trastuzumab who had not developed brain metastases at time of tissue sampling.

The rank of differential expression, probe ID, p-value with respect to global differential expression, t, a moderated t-statistic, B, the log-odds of differential expression between the groups compared, fold change (FC) in MYCN expression when comparing the tumors of patients treated with trastuzumab as to that of patients not treated with trastuzumab, and gene symbol are listed in this chart.

Rank	ID	p-value	t	B	Gene	Gene name
934	28934	7.92E-03	2.70	-2.5277	MYCN	v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog

Table 2: Differential expression of MYCN in primary tumors of breast cancer patients treated with trastuzumab.

The rank of differential expression, probe ID, p-value with respect to global differential expression, t, a moderated t-statistic, B, the log-odds of differential expression between the groups compared, and gene symbol are listed in this chart.

Rank	ID	p-value	t	B	FC	Gene
143	GI_31543215-S-5	0.114767	-1.60594	-4.31	0.80 ± 0.25	MYC

Table 3: Differential expression of MYC in primary tumors of breast cancer patients treated with trastuzumab who had not developed brain metastases at time of tissue sampling.

The rank of differential expression, probe ID, p-value with respect to global differential expression, t, a moderated t-statistic, B, the log-odds of differential expression between the groups compared, fold change (FC) in MYC expression when comparing the tumors of patients treated with trastuzumab as to that of patients not treated with trastuzumab, and gene symbol are listed in this chart.

Rank	ID	p-value	t	B	Gene	Gene name
1159	39735	1.16E-03	-3.4387074	-0.946393	MYC	v-myc avian myelocytomatosis viral oncogene homolog

Table 4: Differential expression of MYC in primary tumors of breast cancer patients treated with trastuzumab.

The rank of differential expression, probe ID, p-value with respect to global differential expression, t, a moderated t-statistic, B, the log-odds of differential expression between the groups compared, and gene symbol are listed in this chart.

Rank	ID	p-value	t	B	FC	Gene
1	GI_4505068-S-3	0.000129	-4.15939	-1.41	0.60 ± 0.20	MXD1

Table 5: Differential expression of MXD1 in primary tumors of breast cancer patients treated with trastuzumab who had not developed brain metastases at time of tissue sampling.

Rank	ID	p-value	t	B	Gene	Gene name
216	26059	1.08E-03	3.34	-0.92661	MXD1	MAX dimerization protein 1

Table 6: Differential expression of MXD1 in primary tumors of breast cancer patients treated with trastuzumab.

The rank of differential expression, probe ID, p-value with respect to global differential expression, t, a moderated t-statistic, B, the log-odds of differential expression between the groups compared, and gene symbol are listed in this chart.

Rank	ID	p-value	t	B	Gene
9472	ILMN_2219767	4.5E-02	-2.0773872	-4.27888	MYCN

Table 7: Differential expression of MYCN in primary tumors of the breast in breast cancer patients following a single treatment with trastuzumab.

The rank of differential expression, probe ID, p-value with respect to global differential expression, t, a moderated t-statistic, B, the log-odds of differential expression between the groups compared, and gene symbol are listed in this chart.

Rank	ID	p-value	t	B	FC	Gene
9	GI_31543215-S-5	0.01484	2.538394	-4.19	1.37 ± 0.37	MYC

Table 8: Differential expression of MYC in primary tumors of breast cancer patients treated with trastuzumab who had not developed brain metastases at time of tissue sampling.

The rank of differential expression, probe ID, p-value with respect to global differential expression, t, a moderated t-statistic, B, the log-odds of differential expression between the groups compared, fold change (FC) in Myc gene expression between the groups compared, and gene symbol are listed in this chart.

Figure 1: MYCN is expressed at significantly higher levels in the primary tumors of trastuzumab-treated breast cancer patients who had not developed brain metastases.

The mRNA expression level of MYCN is graphically depicted in the tumors of patients who had not developed brain metastases, not treated with trastuzumab (left) and in the tumors of patients treated with trastuzumab (right), with mean mRNA expression levels depicted and the result of a statistical test evaluating significance of difference in MYCN mRNA expression level between patients not treated with trastuzumab and patients with trastuzumab, a p -value, listed above.

Figure 2: MYC is expressed at lower levels in the primary tumors of trastuzumab-treated breast cancer patients treated with trastuzumab who had not developed brain metastases.

The mRNA expression level of MYC is graphically depicted in the tumors of patients who had not developed brain metastases, not treated with trastuzumab (left) and in the tumors of patients treated with trastuzumab (right), with mean mRNA expression levels depicted and the result of a statistical test evaluating significance of difference in MYC mRNA expression level between patients not treated with trastuzumab and patients with trastuzumab, a *p*-value, listed above.

Figure 3: MXD1 is expressed at significantly lower levels in the primary tumors of trastuzumab-treated breast cancer patients treated with trastuzumab who had not developed brain metastases.

The mRNA expression level of MXD1 is graphically depicted in the tumors of patients who had not developed brain metastases, not treated with trastuzumab (left) and in the tumors of patients treated with trastuzumab (right), with mean mRNA expression levels depicted and the result of a statistical test evaluating significance of difference in MXD1 mRNA expression level between patients not treated with trastuzumab and patients with trastuzumab, a *p*-value, listed above.

Figure 4: MYC is expressed at significantly higher levels in the primary tumors of trastuzumab-treated breast cancer patients treated with trastuzumab who did develop brain metastases.

The mRNA expression level of MYC is graphically depicted in the tumors of patients who developed brain metastases but not treated with trastuzumab (left) and in the tumors of patients who developed brain metastases and were treated with trastuzumab (right), with mean mRNA expression levels depicted and the result of a statistical test evaluating significance of difference in MYC mRNA expression level between patients not treated with trastuzumab and patients with trastuzumab, a *p*-value, listed above.